## REMARKS

Claims 52-85 are pending. Favorable reconsideration is respectfully requested.

At the outset, Applicants would like to thank Examiner Leffers for indicating that the claims are free of the prior art and for helpful suggestions regarding claim amendments.

The rejection of the claims under 35 U.S.C. §112, second paragraph, is believed to be obviated by the amendment submitted above in part and is, in part, respectfully traversed.

Claim 52 has been amended as suggested by the Examiner.

Claim 53 has been amended to clarify that the culturing recited therein follows the culturing recited in Claim 52.

Claims 61, 63, 68, 70, and 85 have been amended for clarity.

Claim 75 has been amended to specify producing a supernatant and a cell pellet in (b).

Claim 85 recites functionalizing the isolated protein. Such a procedure is described in detail in the specification at page 10, lines 22-30, page 14, lines 15-35, and page 15, lines 1-28. In view of that description, one would readily appreciate the meaning of functionalizing the protein.

Based on the foregoing, Applicants submit that the claims are definite within the meaning of 35 U.S.C. §112, second paragraph. Accordingly, withdrawal of this ground of rejection is respectfully requested.

The rejection of Claims 69-72 and 74 under 35 U.S.C. §112, first paragraph, is respectfully traversed.

The present invention implicitly requires mutated (*in vivo*) aminoacyl tRNA synthestases for obtaining mutated target proteins and therefore, the method of the invention for selecting cells also allows the *in vivo* diversification of the natural tRNA synthetases.

However, the mutations of the tRNA synthetases have no direct effect on the life and the growth of the cells. The mutations which have such an affect are the mutations

introduced in any other target proteins whose functions are essential for the survival of the cells.

These two types of mutations are discussed below.

**(1) Amino Acyl tRNA Synthetases Mutations**

These mutations are produced *in vivo* because of the method of selection. The mutations allow the tRNA synthetases of the selected cells to enlarge the loading of amino acids in the mutated target codons, such that the tRNA synthetases carry either a non-conventional amino acid or a conventional amino acid, which however is different of the amino acid which is found in the same position in the native protein.

They are not, as such, essential for the survival of the cell. The precise knowledge of these mutations is not necessary for implementing the invention.

The present specification clearly discloses that it is not necessary, for implementing the method of selection of viable stable cells, to know in advance the mutation of the aminoacyl tRNA synthetase of the cells.

**(2) Missense Mutations Introduced in the Target Codon**

Conversely, these mutations are mutations of the target codon of a gene which is essential for the life of the cell.

The introduction of at least one of these types of mutations cancels or decreases the vital functions of the protein expressed by this essential gene, and in case of absence of the selective culture medium of the invention, such mutations produce the death of the cell.

These mutations are well-defined in the specification. A missense mutation introduced may be either the introduction of an amino acid other than the amino acid incorporated by ribosomes during the biosynthesis of proteins synthesized by prokaryotic or eukaryotic organisms or the introduction of any amino acid incorporated in place of the

amino acid which is normally incorporated at this site with regard to the translated nucleic acid sequence (see page 2, lines 31-39). These mutations are introduced at the level of a target codon of the gene coding for the protein. The introduction of a missense mutation in a target protein was known when the present application was filed. For example, a missense mutation may be made by site-specific mutagenesis.

The specification provides to the one skilled in the art all the teaching for introducing these missense mutations in any target protein. For example, the specification refers to Kunkel TA, Roberts JD, Zakour RA, *Rapid an efficient site-specific mutagenesis without phenotype selection*, Methods Enzymol. 1987, 154, 367-82. In connection therewith, please find enclosed a publication of Döring V and al. wherein new results obtained by the present invention are described.

Based on the foregoing, Applicants submit that the written description requirement is satisfied. Accordingly, withdrawal of this ground of rejection is respectfully requested.

Applicants submit that the present application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully Submitted,

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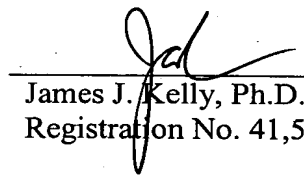
Norman F. Oblon



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# Enlarging the Amino Acid Set of *Escherichia coli* by Infiltration of the Valine Coding Pathway

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Aminoacyl transfer RNA (tRNA) synthetases establish the rules of the genetic code by catalyzing the aminoacylation of tRNAs. For some synthetases, accuracy depends critically on an editing function at a site distinct from the aminoacylation site. Mutants of *Escherichia coli* that incorrectly charge $tRNA^{Val}$ with cysteine were selected after random mutagenesis of the whole chromosome. All mutations obtained were located in the editing site of valyl-tRNA synthetase. More than 20% of the valine in cellular proteins from such an editing mutant organism could be replaced with the noncanonical aminobutyrate, sterically similar to cysteine. Thus, the editing function may have played a central role in restricting the genetic code to 20 amino acids. Disabling this editing function offers a powerful approach for diversifying the chemical composition of proteins and for emulating evolutionary stages of ambiguous translation.

The chemical invariance of the 20–amino acid building blocks of proteins is well established. The only known extensions to this invariant set are formyl-methionine (*1*) and selenocysteine (*2*), both incorporated in response to punctuation signals during translation in certain organisms. Thus, although species have colonized dissimilar terrestrial habitats throughout geologically recorded time, this diversification has not been mirrored in the evolution of organisms to adopt specialized sets of amino acids. For instance, thermophilic, mesophilic, and psychrophilic organisms all assemble proteins by combining the same types of 20 canonical amino acids into different protein sequences. Standing as the "missing link" between alanine and valine (*3*), aminobutyrate (Abu, also known as butyrine) can be generated by transamination from the physiological metabolite 2-oxo-butyrate and should thus be considered a latent metabolite (*4*). Its absence is therefore particularly conspicuous in the proteins of extant organisms.

The selection of amino acids for protein synthesis is done by aminoacyl tRNA synthetases. Typically, each of 20 synthetases catalyzes the attachment of its cognate amino acid to the 3′ end of its cognate tRNA, and amino acids are, in this way, associated with specific triplets of the genetic code (*5*). The active site of several of these enzymes inherently lack the capacity to discriminate between closely similar amino acids at a level sufficient to explain the high accuracy of the code. For that reason, a given enzyme may misactivate amino acids that are closely similar (in size and shape) at a low frequency (0.1 to 1%) (*6*). To correct these errors, in many cases, a hydrolytic editing function, at a separate active site, has developed (*7–10*). One example of a synthetase that has editing activity is valyl-tRNA synthetase (ValRS), which misactivates the isosteric natural amino acid Thr (*9*), as well as the unnatural Abu (*11*). Misactivation of these amino acids leads to transient mischarging of $tRNA^{Val}$, followed by hydrolytic deacylation (editing) of the mischarged amino acid from the tRNA.

In the work reported here, we aimed to establish conditions of artificial selection that promoted usage of noncanonical amino acids, such as Abu, that were not accessed or retained by natural selection. Others are attempting to incorporate a noncanonical amino acid into a protein by introducing a foreign, "orthogonal" synthetase-tRNA pair that can insert the amino acid at a specialized stop codon (*12–14*). In a different approach we set out to enlarge the chemistry of translation by having a noncanonical amino acid "infiltrate" codons normally specific for one of the natural amino acids. By assigning two amino acids (a cognate and a noncognate) to a single codon so as to provide a selective advantage to the reprogrammed cells, global changes in the amino acid compositions of all cellular proteins could be made.

A direct selection for restoring an enzymatic activity through incorporation of Abu could not be easily set up, because its aliphatic side chain lacks chemical reactivity and therefore cannot act as a catalytic residue. We thus resorted to an indirect scheme based on the structural resemblance of Abu with Cys (Fig. 1A), an essential catalytic residue in numerous enzymes. Selecting a synthetase mutant that mischarged its cognate tRNA with Cys might result in Abu being mischarged by the mutant synthetase.

We took advantage of the *thyA* conditional selection screen in *E. coli*, based on the absolute requirement for an active thymidylate synthase when thymidine is not provided as a growth factor (*15*). This same screen was used previously to assess the potency of suppressor Cys-tRNAs in codon misreading (*16*) and to enforce phenotypic suppression by the noncanonical azaleucine (*17*). An entire set of plasmid-borne *thyA* alleles with all 64 different codons at position 146 was constructed for altering the catalytic site occupied by an essential Cys (*18*, *19*). Each allele was tested for its ability to restore growth to an *E. coli* strain (lacking the chromosomal copy of *thyA*) on mineral glucose medium in the absence of thymidine (*16*, *17*). Three of the 64 plasmid-borne *thyA* alleles restored growth. These had one of three codons: UGU, UGC, or UGA. The growth responses of the UGU and UGC alleles were expected, as these code for cysteine. The positive response of UGA (a termination codon in *E. coli*) likely results from read-through by Cys-tRNA, and thus demonstrates the sensitivity of the selection assay.

Strains bearing inactive alleles of *thyA* were then tested to see if they could be suppressed by supplying them with excess L-cysteine in mineral medium devoid of thymidine. Shallow growth was reproducibly observed on L-cysteine gradient plates (*20*) for the missense alleles having any of the eight codons AUN and GUN alone. Growth was stronger with alleles bearing any of the four Val codons (GUU, GUC, GUA, and GUG) than for those with the three Ile codons (AUU, AUC, and AUA) or for the Met codon AUG. Cysteine suppression of the three Ile codon–bearing alleles and of the Met codon–bearing allele was abolished by addition of exogenous L-isoleucine and L-methionine, respectively. Suppression of the four Val codon–bearing alleles was abolished by addition of exogenous L-valine plus L-isoleucine but not by L-isoleucine alone (*20*). The four $Val^{146}$ alleles gave a similar growth response in cysteine gradient plates, despite being decoded by three different $tRNA^{Val}$ isoacceptors, thus suggesting that Cys is be-

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ing mischarged onto all three Val isoacceptor tRNAs by ValRS. Altogether, these results suggested that ValRS catalyzed the formation of Cys-tRNA$^{Val}$ in vivo at a rate sufficient for active thymidylate synthase production and that this mischarging reaction was prevented by increasing the intracellular concentration of L-valine. This interpretation is in line with earlier reports of Cys misactivation by ValRS in vitro (*11*).

Suppression of *thyA:Val146* alleles was weak on plates and required high L-cysteine concentrations (developed from a gradient starting at a concentration of 100 mM). It could thus be anticipated that a scarce L-cysteine supply should select for an enhanced efficiency of phenotypic suppression. Two experimental procedures were followed to this end. In the first procedure strain β5519 [*thyA::erm*$^+$ Δ*nrdD::kan*$^+$ pTS13 (*bla*$^+$ *thyA*:146GUA)] was propagated over 100 generations in serial liquid culture with limiting cysteine (1.5 mM) under anaerobic conditions, resulting in isolation of strain β5420 (*21*). The second procedure relied on a one-step selection under aerobic conditions on plates containing a nonoxidizable precursor that is inefficiently converted into cysteine by *E. coli*, *S*-carbamoyl-cysteine (Scc) [Fig. 1A and (*21*)]. A mutator allele (*dnaQ* or *mutS*) was introduced into the test strain β5519 to increase the frequency above $10^{-10}$ of Scc-suppressible thymidine auxotrophs (*21*). This approach resulted in the isolation of four strains: β5456, β5479, β5485, and β5486. All five isolated strains could not grow at 42°C, in agreement with the heat sensitivity generally caused by translational errors (*22*). The cysteine-suppression phenotype is shown for one of these strains (β5456) in Fig. 1B, together with its abolition by L-valine (supplied as an Ile-Val peptide).

Judging from these phenotypes, mutations in the *valS* gene for valyl-tRNA synthetase were suspected for each isolated strain. To test for this possibility, we took advantage of the *nrdD::kan*$^+$ marker (*21*) located 0.4 min from *valS* on the *E. coli* chromosome. For the five mutants, the trait of cysteine- or Scc-suppressible thymidine auxotrophy was indeed found to cotransduce with the *nrdD::kan*$^+$ marker in a proportion of about 45%. Further characterization of the *valS* mutations was performed by polymerase chain reaction (PCR) amplification followed by sequencing of five *nrdD::kan*$^+$ transductants exhibiting Scc-suppressible thymidine auxotrophy derived from the five isolated strains (*23*). As shown in Table 1, each of the five mutants contained a single–amino acid substitution at positions within the conserved editing domain (known as CP1) of ValRS (*24*). The following mutations were identified: T222P, R223H, D230N, V276A, and K277Q. Remarkably, two of these positions (T222 and D230) align with conserved posi-

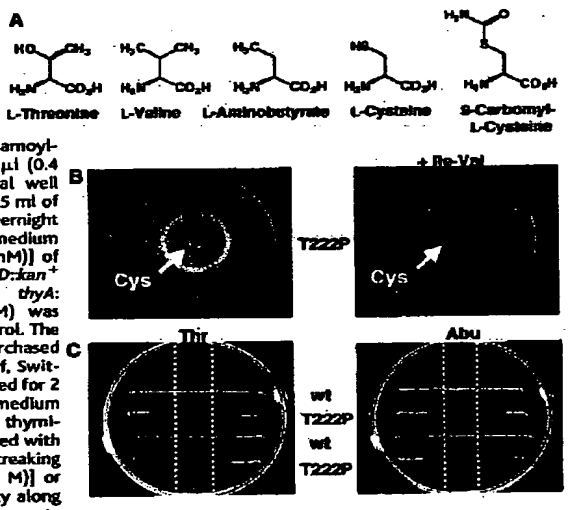


**Fig. 1.** Suppression and toxicity phenotypes. Amino acid gradient plates were prepared using minimal medium (29). (**A**) Structures of threonine, valine, α-aminobutyric acid, cysteine, and *S*-carbamoyl-cysteine. (**B**) Cysteine [100 μl (0.4 M)] was loaded in a central well after spreading and drying 0.5 ml of a 5/1000 dilution of an overnight culture [in minimal glucose medium containing thymidine (0.3 mM)] of β5456 [*thyA::erm*$^+$ Δ*nrdD::kan*$^+$ *valS:T222P* pTS13 (*bla*$^+$ *thyA*:146GUA)]. Ile-Val (0.3 mM) was added in one plate as a control. The dipeptide Ile-Val was purchased from Bachem AG (Bubendorf, Switzerland). Plates were incubated for 2 days at 30°C. (**C**) Minimal medium plates supplemented with thymidine (0.3 mM) were pretreated with amino acid solutions by streaking either with Thr [50 μl (0.2 M)] or Abu [50 μl (0.1 M)] vertically along the diameter of the plate to create an amino acid gradient. Mutant β5456 and wild-type β5419 [*thyA::erm*$^+$ Δ*nrdD::kan*$^+$ pTS13 (*bla*$^+$ *thyA*:146GUA)] strains were then streaked horizontally across the plates and incubated for 2 days at 37°C.

**Table 1.** Mutations of the *valS* gene selected by suppression of the thymidine auxotrophy of strain β5419 [Δ*thyA::erm*$^+$ Δ*nrdD::kan*$^+$pTS13 (*bla*$^+$ *thyA*:146GUA)]. The wild-type codons and amino acids and the corresponding changes in the mutant strains are in bold type.

| Strain | Method of isolation | Mutator genotype | Codon of *valS* | | | | |
|---|---|---|---|---|---|---|---|
| | | | 222 | 223 | 230 | 276 | 277 |
| β5419 | – | wt | ACC Thr | CGT Arg | GAT Asp | GTG Val | AAA Lys |
| β5456 | Selection on Scc | Δ*dnaQ* | CCC Pro | CGT | GAT | GTG | AAA |
| β5479 | Selection on Scc | Δ*mutS* | ACC | CAT His | GAT | GTG | AAA |
| β5486 | Selection on Scc | Δ*mutS* | ACC | CGT | AAT Asn | GTG | AAA |
| β5485 | Selection on Scc | Δ*mutS* | ACC | CGT | GAT | GCG Ala | AAA |
| β5520 | Selection on Cys under anaerobiosis | wt | ACC | CGT | GAT | GTG | CAA Gln |

**Table 2.** Incorporation of Abu into cells bearing wild-type and T222P mutant alleles of *valS*. Cultures of the Δ*ilv* strain CU505 (27) and the isogenic strain β5498 carrying the *valS:T222P* allele were grown overnight in minimal medium (29) with Ile-Leu (0.3 mM) and limiting valine (0.04 mM Ile-Val), diluted (1/2), and adjusted with Ile-Val (0.02 mM) with or without Abu (0.2 mM). After 24 hours of growth, total protein was extracted as follows. Cells were first harvested by centrifugation and washed in cold 10% trichloroacetic acid (TCA, 1/2 of the culture volume). Cells were then recentrifuged at 4000*g* for 10 min, resuspended in cold 10% TCA (1/10 of the culture volume) and centrifuged again. The washed cells were resuspended in 5% TCA, heated at 95°C for 30 min, and centrifuged (4000*g*, 10 min). The precipitate was washed three times with cold acetone and dissolved in 50 mM $NH_4HCO_3$. Proteins were hydrolyzed in 6 M HCl–0.2% phenol at 110°C for 20 hours in sealed tubes. Norleucine was added as an internal standard. Aliquots of the hydrolysates were analyzed on a Beckman 6300 amino acid analyzer. Amino acids were quantified by appropriate standards and values are presented relative the wild-type (WT) control that lacked Abu.

| Amino acid incorporated | WT | T222P | WT + Abu | T222P + Abu |
|---|---|---|---|---|
| Abu | 0.0 | 0.0 | 0.0 | 0.24 |
| Val | 1.0 | 0.95 | 1.0 | 0.73 |
| Val + Abu | 1.0 | 0.95 | 1.0 | 0.97 |
| Ile | 1.0 | 1.0 | 1.0 | 1.0 |
| Ala | 1.0 | 0.97 | 1.0 | 0.92 |

tions in IleRS that had been demonstrated previously to be involved in the hydrolytic editing of misacylated Val-tRNA$^{Ile}$ (*25*) (Fig. 2A).

ValRS is known to misactivate Thr and to generate Thr-tRNA$^{Val}$, which normally is hydrolyzed by the ValRS editing activity (*9*). If the ValRS mutants in Table 1 are impaired for editing, then strains bearing each of these mutations should misincorporate Thr into protein, and L-threonine would then be toxic in these strains. The growth of the five different *valS* strains was therefore tested in the presence of L-threonine. All displayed high sensitivity toward exogenous L-threonine (at 2 mM), whereas the parent strain β5419 was insensitive to L-threonine at all concentrations. The results for the strain carrying the *valS:T222P* allele are shown in Fig. 1C.

The phenotype of the *valS:T222P* allele suggested that the T222P enzyme mischarged tRNA$^{Val}$ with Thr and Cys in vivo. The T222P mutant enzyme was, therefore, expressed and partially purified so that it could be directly assayed for the ability to misacylate tRNA$^{Val}$. The purified enzyme had the same activity as the wild-type enzyme for charging with valine (Fig. 2B, inset). In contrast, the T222P mutant enzyme misacylated tRNA$^{Val}$ with Thr to give Thr-tRNA$^{Val}$ whereas the wild-type enzyme produced no detectable mischarged tRNA$^{Val}$ (Fig. 2B). Misacylation of tRNA$^{Val}$ with cysteine was also catalyzed only by the mutant enzyme.

We anticipated that ValRS mutants that misincorporated Cys would also misincorporate Abu. Indeed, the strain carrying the *valS:T222P* allele on the chromosome (β5456) was sensitive to Abu (Fig. 1C) (whereas its wild-type counterpart was not), suggesting incorporation of Abu in response to Val codons. With this in mind, we showed that Abu could contribute to the relief of L-valine auxotrophy of the Δ*ilv* strain CU505, but only in the presence of the *valS:T222P* allele (*21*). Whole-cell protein was isolated from strain CU505 grown in the presence of Abu (0.2 mM), in the presence or absence of the *valS:T222P* allele in the host cell. Analysis of amino acid composition showed that 24% of the valine was replaced by Abu only in the strain harboring the mutant allele (Table 2). Finally, the valine-rich yeast protein AlaXp (swissprot:P53960) was overexpressed and purified from strains containing the *valS:T222P* allele grown in the presence of Abu. The protein samples were digested by trypsin and analyzed by mass spectrometry. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis showed that, when AlaXp was produced in the strain carrying the T222P mutation, it contained a mixture of Val and misincorporated Abu (Fig. 2C). For a given peptide the degree of misincorporation ranged between 9.5% and 18% per Val codon. Sequencing of several Abu-containing peptides confirmed that Abu was specifically misincorporated into positions designated by Val codons.

Thus, *E. coli* strains that proliferate only because of infiltration of the Val coding pathway were selected, and all contained mutations leading to single–amino acid substitutions in the editing site of ValRS. This observation is consistent with a central role for editing in restricting the genetic code to 20 amino acids, by preventing the invasion of other amino acids such as Abu. Indeed, the editing sites in IleRS and ValRS are rigorously conserved in even the most deeply branched organisms in the tree of life. However, the translation accuracy maintained by editing may prevent further chemical evolution of proteins. Thus, disabling the editing function of a synthetase, as demonstrated in the present work, offers a powerful approach to diversify the chemical composition of proteins produced in vivo.

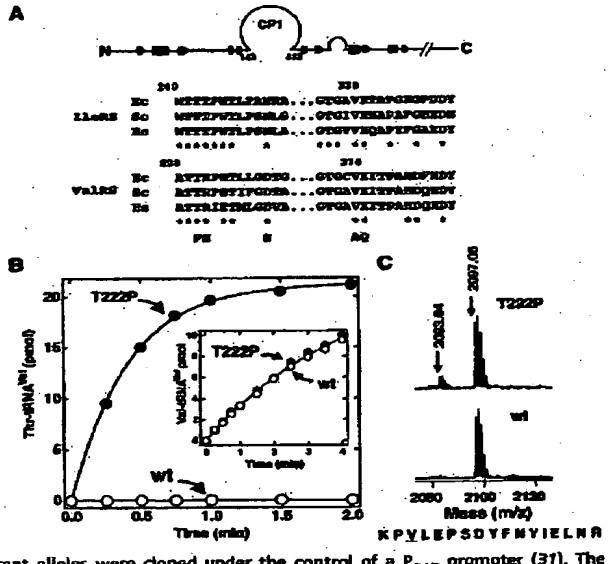


**Fig. 2.** Point mutations in the editing site and their consequences. (**A**) Positions of the five point mutations isolated in the editing site of ValRS are shown. The IleRS editing site (CP1) (*30*, *31*) that intersects the alternating β strands (pentagons) and α helices (rectangles) of the catalytic domain is shown. Alignment of residues in the editing sites of IleRS and ValRS is also shown, with the strictly conserved residues among all published sequences labeled with a colon. Abbreviations are Ec, *E. coli*; Sc, *Saccharomyces cerivisiae*; Hs, *Homo sapiens*. (**B**) Misaminoacylation of tRNA$^{Val}$ with Thr by the T222P mutant enzyme at pH 7.5 and 37°C. The wild-type (wt) and mutant alleles were cloned under the control of a $P_{BAD}$ promoter (*31*). The enzymes were partially purified from a laboratory strain lacking the chromosomal copy of the *valS* gene (Δ*valS::kan*$^+$). The purification and aminoacylation procedures were adapted from Hendrickson *et al.* (*33*). (Inset) Aminoacylation of tRNA$^{Val}$ with Val by the two enzymes. (**C**) In vivo incorporation of aminobutyrate. The His-tagged protein AlaXp was expressed in two Δ*ilv* strains containing the wild-type *valS* or the mutant *valS:T222P* allele, in MS medium containing Ile-Leu (0.3 mM), Ile-Val (0.02 mM), and Abu (0.2 mM). AlaXp was purified with Ni-NTA agarose (Qiagen), cut out of an SDS–polyacrylamide gel electrophoresis (SDS-PAGE) preparative gel, and prepared for MALDI-MS and liquid chromatography–tandem mass spectrometry. (μ-LC-MS/MS) mass analysis (*34*). The MALDI-MS analyses were performed in a Voyager-Elite time-of-flight mass spectrometer with delayed extraction (PerSeptive Biosystems, Framingham, Massachusetts). The spectrum for peptide Lys$^{156}$ to Arg$^{172}$ with mass 2097.04 is shown on the bottom panel (wild-type cells). The top panel shows the peptide resolved into two components when isolated from cells bearing the T222P allele of the gene for ValRS. The second component has a mass of 2083.04, exactly 14 mass units less then the "wild-type" peptide. Multiple peaks correspond to $^{13}C$ isotopic forms that separate peptides differing by mass units of 1, 2, 3, etc.


**References and Notes**

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19. The 64 alleles of *thyA* with different codons at position 146 of the coding sequence were constructed as follows. First, a unique Nhe I site was introduced through a $Gly^{429} \rightarrow Ala$ substitution in the *thyA* coding region by site-directed mutagenesis (26) of plasmid pTSO (16) to yield plasmid pTSO1. Oligonucleotides THY1 (5′-CTGGATAAATGGCGCTAGCACCGTGCCATGCATTC-3′) and THY2 (5′-TCTGCCACATAGAACTGGAAGAATGCATGGCACGGT-3′) were used for this mutation, which preserved the sense of the codon thus mutated. Plasmid pTSO1 was then digested with Nhe I and Nsi I to remove from the *thyA* coding region an 18–base pair fragment containing codon 146 (UGC). All 64 oligonucleotides of the *thyA* coding sequence from nucleotides 427 to 444 and the 64 oligonucleotides of the partial reverse sequence were constructed (GENAXIS Biotechnology, Montigny le Bretonneux, France). The 64 pairs of complementary oligonucleotides were annealed and ligated with the digested plasmid pTSO1.
20. Cysteine gradient plates were generated as described in the legend to Fig. 1B. The effects of L-valine alone could not be directly examined because exogenous L-valine is known to inhibit growth of *E. coli* K12 in minimal medium (27). This inhibition is relieved if L-isoleucine is also supplied. Thus, the Ile-Val dipeptide was used as a valine source, because this dipeptide is transported across the cell membrane and then broken down to isoleucine and valine (28).
21. Supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/292/5516/501/DC1.
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23. *Escherichia coli* chromosomal DNA was extracted using a DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) following the instructions of the manufacturer. PCR to amplify the *valS* gene was performed as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, annealing at 57°C for 30 s, and primer extension at 72°C for 200 s. The final step was a primer extension at 72°C for 600 s. The reaction was carried out using 2 units of Vent DNA polymerase (New England Biolabs, Beverly, MA) and 100 ng of chromosomal DNA in a 100-μl reaction mixture. The following primers were used: VAL1 (5′-GGGGAATTCGGTGTGTGAAATTGCCGCAGAACG-3′), and VAL2 (5′-GGCAAGCTTTCAGGTATTTGCTGCCCAGATCGA-3′). Two independent PCR amplification products of each mutant were sequenced (GENAXIS Biotechnology).
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# Cooperation and Competition in the Evolution of ATP-Producing Pathways

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Heterotrophic organisms generally face a trade-off between rate and yield of adenosine triphosphate (ATP) production. This trade-off may result in an evolutionary dilemma, because cells with a higher rate but lower yield of ATP production may gain a selective advantage when competing for shared energy resources. Using an analysis of model simulations and biochemical observations, we show that ATP production with a low rate and high yield can be viewed as a form of cooperative resource use and may evolve in spatially structured environments. Furthermore, we argue that the high ATP yield of respiration may have facilitated the evolutionary transition from unicellular to undifferentiated multicellular organisms.

Heterotrophic organisms obtain their energy by the degradation of organic substrates into products with lower free energy. The free energy difference between substrate and product can in part be conserved by production of ATP and in part be used to drive the degradation reaction. The maximal ATP yield is obtained if the entire free energy difference is conserved as ATP. However, in this case the reaction is in thermodynamic equilibrium, and therefore the rates of substrate degradation and ATP production vanish. If some of the free energy difference is used to drive the reaction, the rate of ATP production increases with decreasing yield until a maximum is reached. Hence, for fundamental thermodynamic reasons there is always a trade-off between yield (moles of ATP per mole of substrate) and rate (moles of ATP per unit of time) of ATP production in heterotrophic organisms (*1*–*4*).

A trade-off between yield and rate of ATP production is also present in sugar degradation by fermentation and respiration. In the presence of oxygen and sugars, many organisms are in principle capable of using both pathways to produce ATP. Because the ATP production rate of respiration is rapidly saturated at high levels of resource or limited oxygen supply (*5*–*8*), these organisms can choose, at least in the evolutionary sense, to increase the rate of ATP production by using fermentation in addition to respiration. However, because the yield of fermentation is much lower than that of respiration (2 mol versus about 32 mol of ATP per mole of glucose), the use of fermentation in addition to respiration increases the rate of ATP production at the cost of a lower total yield.

If energetic limitation is an important factor for organisms in their natural environment, we then expect that the properties of ATP-producing pathways have been under strong selection pressure during evolution. The existence of a trade-off between yield and rate of ATP production leads to the following question: Under what conditions is it favorable to use a pathway with high yield but low rate, as opposed to a pathway with low yield but high rate? A cell using a pathway with high yield and low rate can produce more ATP (and thus more offspring) from a given amount of resource. However, this advantage disappears when the cell is in resource competition with cells that produce ATP at a higher rate but a lower yield. While only those cells that consume the resource more rapidly benefit from the higher rate of ATP production, all competitors exploiting the resource share the consequences of the more rapid resource exhaustion (*9*).

The competition between cells with different properties in ATP production can be illustrated with a simple population biological model. Assume that a resource $S$ is produced at a constant rate $\nu$ and is consumed by $n$ different strains of cells, $N_i$, at a rate of $J_i^S(S)$ per cell. We assume that the growth rates of the strains are energetically limited and proportional to the rate of ATP produc-

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